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Utilization of antisense oligonucleotides to study the role of 5-cytosine DNA methyltransferase in cellular transformation and oncogenesis

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Abstract

A large body of data point toward 5-cytosine DNA methyltransferase 1 (DNMT1) as a critical component of oncogenic programs. The study of the role of DNMT1 in cancer has been hindered by the lack of specific inhibitors. A different approach to study the role of DNMT1 in cancer is to use sequence-specific antisense oligonucleotides against DNMT1 mRNA. This paper discusses methods used to identify sequence-specific antisense oligonucleotides and to assess their DNA methylation inhibitory properties. Antisense oligonucleotides are applied to determine whether DNMT1 plays a causal role in specific cancer models ex vivo as well as in vivo.

Keywords: DNA methylation; Antisense; DNA methyltransferase; Nearest-neighbor analysis; Tumorigenesis; Soft agar assays; Anchorage-independent growth

1. Introduction

Methylation of specific CpG sequences in mammalian DNA is emerging as an important mechanism of regulation of genomic functions such as differential control of gene expression [1,2]. DNA methylation is strongly correlated with diverse facets of differential gene expression such as the allele-specific expression observed in X-linked genes [3,4] and parental imprinting [5], as well as tissue-specific gene expression [1,2]. The discovery of methylated DNA-binding proteins (MBDs) such as MeCP2 [6] has provided a molecular explanation to the well-established observation of a tight linkage between inactive chromatin and DNA methylation [7]. Methylated DNA attracts MBDs, which in turn recruit histone deacetylases to genes. Deacetylation of histone tails is believed to be responsible for the closed configuration of inactive chromatin. A number of observations have demonstrated synergism between histone deacetylase inhibitors and DNMT inhibition in inducing the expression of genes that are silenced in cancer such as tumor suppressor genes [8].

Because DNA methylation can affect a large number of genomic programs, it stands to reason that alterations in the DNA methylation machinery might be involved in the genesis of oncogenic schemes [9]. A large body of evidence has indicated that alterations in DNA methylation activity [10] and DNA methylation patterns [11] occur in cancer cells. The directions that these changes in DNA methylation patterns take is somewhat confusing. Both global hypomethylation [12] and hypermethylation [13] of specific genes have been observed. Hypermethylation in cancer cells has, however, received special attention since it seems to be localized to regulatory regions of tumor suppressor genes [14]. What is the role that these changes in methylation might play in cancer?

Hypermethylation might be a mere reflection of the inactive state of certain tumor suppressor genes in cancer cells or might be the cause of inactivation. Even if hypermethylation of tumor suppressor plays a causal role in cancer cells, it is possible that methylation of tumor suppressors is a random event, which is selected

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by the oncogenic process because of its growth-promoting effects. Alternatively, changes in methylation of tumor suppressor genes might be a critical component of some or all oncogenic pathways. In addition to the specific changes in DNA methylation, DNMT1 is also induced in many tumors [15]. It is not clear whether the induction of DNMT1 is responsible for hypermethylation of tumor suppressor genes, and several observations indicate no correlation between methylation of tumor suppressor genes and the cellular levels of DNMT1 (for a review see [16]). However, it is now becoming clear that DNMT1 can silence genes and specifically tumor suppressor genes by a mechanism that does not involve DNA methylation [17]. Inhibition of DNMT1 has been shown to arrest the growth and DNA synthesis of cancer cells [18]; however, the mechanism is not clear as of yet.

Specific DNMT1 inhibitors are required to study the mechanisms through which DNMT1 is involved in cellular transformation. It is clear that to determine whether DNMT1 plays a causal role in the genesis of different cancer models, it is critical to use inhibitors of DNMT1. Inhibitors of DNMT1 could also be used to determine whether increased methylation represses the expression of tumor suppressor genes and to identify these putative genes by differential display. The only DNMT1 inhibitor that is broadly used is 5-azacytidine (5-aza-CdR) [19]. Whereas 5-aza-CdR is used in vivo [20] and in vitro [21] to study the role of DNMT1 in cancer and tumor suppressor gene expression, it has nonspecific antimitotic and toxic effects (reviewed in [22]). 5-Aza-CdR is a nucleoside analog that is incorporated into the DNA and covalently binds and traps DNMT1 [23]. 5-Aza-CdR might exert its antimitotic and toxic effects by trapping bulky DNMT1 protein onto DNA and not by inhibiting DNMT1 [24].

An alternative approach that has recently been demonstrated to inhibit DNMT1 is using either ectopically expressed antisense RNA [25,26] or antisense oligonucleotides directed against the DNMT1 mRNA [27,28]. Antisense oligonucleotides have been developed against a growing number of mRNAs and have been shown to be effective and specific inhibitors of many genes in culture and in vivo [29]. However, as is the case with any class of inhibitors, nonspecific effects of oligonucleotides confounded the interpretation of antisense studies [30]. Proper design of antisense experiments requires that special attention be given to the selection of a *bona fide* sequence-specific antisense and the design of the appropriate controls [30]. The delivery of antisense to cells in culture is inconsistent and different methods have been developed to allow for efficient intracellular delivery [30]. Once these measures have been taken, antisense oligonucleotides could be potent and efficacious inhibitors of gene expression. This paper describes the methods used in my laboratory to select and ascertain the specificity of DNMT1 antisense oligonucleotides, to determine its DNMT and DNA methylation inhibitory effects, and to determine the effects that inhibition of DNMT1 has on cellular transformation in culture and in vivo [27,28].

2. Selection of DNMT1-specific antisense oligonucleotides

We routinely use for our cell culture and in vivo animal studies 18- to 20- nucleotide-long phosphorothioate-modified deoxyoligonucleotides. Although some side effects of phosphorothioate-modified oligonucleotides have been observed in clinical trials, sequence-specific and potent antisense phosphorothioate-modified oligonucleotides are good inhibitors in animals and in cells in culture [29]. The phosphorothioate modification renders oligonucleotides resistant to nucleases and therefore increases their potency and stability [31]. Phosphorothioate-modified phosphoramidate nucleotides are commercially available and most commercial oligonucleotide synthesis facilities can synthesize phosphorothioate oligonucleotides. However, since nonspecific effects of the phosphorothioate-modified oligonucleotides were observed in vivo, chimeric oligonucleotides that bear a combination of backbone modifications were introduced.

One chimeric oligoucleotide that is commonly used has four nucleotides at both the 3′ and 5′ ends whose backbone is modified at the ribose by an *O*-methyl modification while the core nucleotides backbone is phosphorothioate modified. The *O*-methyl modification reduces the polarity and the nonspecific protein binding activity, and increases the stability of the oligonucleotide and hybrid stability [32]. However, this modification inhibits RNase H activity, which is believed to be responsible for the degradation of the mRNA–antisense hybrid. The phosphorothioate modification does not inhibit RNase H; therefore, the phosphorothioate-modified core directs RNase H activity toward the mRNA–antisense hybrid. We are currently using such a chimeric antisense oligonucleotide directed against human DNMT1 [MG88] [28]. Another chimeric DNMT1 antisense oligonucleotide, MG98, is currently in phase II clinical trials as an anticancer agent. The oligonucleotides used in my laboratory are purified by the oligonucleotide synthesis facility on HPLC using a phenyl-Sepharose column followed by DEAE 5PW anion-exchange chromatography.

Since phosphorothioate-modified oligonucleotides exhibit, in our hands, nonspecific effects, including effects on cell growth at high doses [500 nM to 1 μM with lipid carriers or 10–20 μM in the absence of a carrier), it is critical that a potent antisense sequence is selected. Ideally, an antisense that inhibits DNMT1 mRNA with

an EC_{50} of 10–50 nM will be well within a therapeutic window where nonspecific effects of the oligonucleotides are undetectable. A reliable test for a specific DNMT1 antisense agent is that it reduces the levels of DNMT1 mRNA in the cell as determined by either Northern blot, RNase protection analysis, or competitive polymerase chain reation (PCR) (as will be detailed below).

We have found no clear rule as to which sequence in an mRNA is a hot spot for antisense inhibition. Most sequences that we have tested had some antisense activity. However, in our hands both the 5′ region around the second ATG site and 3′ UTR regions are very sensitive. Other potential targets are intron–exon boundaries [33]. Probably the safest approach is to screen a number of sequences complementary to the 5′ and 3′ UTR of DNMT1 mRNA. A good published example of such a screening protocol is the one applied for selecting an anti-*c-raf* antisense oligonucleotide [34]. The different oligonucleotides to be screened should be applied onto cells in culture once the delivery lipid has been selected, and their effect on DNMT1 mRNA should be determined as will be described below.

A critical point in selecting an antisense oligonucleotides is demonstrating that the control nonspecific oligonucleotide has no effect on the target mRNA. We have previously used two kinds of controls for nonspecific effects: a scrambled sequence and a reverse sequence. Another kind of control that is widely used is an oligonucleotide containing three base pair mismatches to the cognate sequence. Some sequence elements have been previously reported to exhibit non-antisense-related effects such as CG sequences [35] and G quartets [36] and should be avoided if possible.

Using this approach, we have previously identified in collaboration with Hybridon a potent mouse DNMT1 antisense oligonucleotide [$EC_{50} < 50$ nM] (HYB1015 84), 5′ TCT ATT TGA GTC TGC CAT TT 3′, corresponding to bases −2 to +18 in the murine DNMT1 mRNA (relative to the second ATG translation initiation site) [27]. The scrambled control of this oligonucleotide is (HYB 102277) 5′ TGT GAT TCT CCT TAT TCG AT 3′, and the reverse sequence is: (HYB101585) 5′ TTT ACC GTC TGA GTT TAT CT 3′. Potent human antisense DNMT1 oligonucleotides have been developed in collaboration with MethylGene using the approach described above. The human DNMT1 antisense sequence used in my laboratory is 5′ AAG CAT CAG CAC CGT TCT CC 3′ (MG88), and as a control we use 5′ AAC GAT CAG GAC CCT TGT CC 3′ (MG208). Antisense oligonucleotides are species specific because of interspecies differences in the nucleotide sequence of DNMT1. However, the approach described here for selecting antisense oligonucleotides could be applied to any species, once the nucleotide sequence of the cDNA encoding DNMT1 mRNA is known.

3. Cellular delivery of antisense oligonucleotides

A critical aspect of antisense oligonucleotide treatment is the cellular delivery of the antisense oligonucleotides. Before setting up an antisense experiment with a specific cell line(s), delivery has to be optimized. The efficacy of delivery of antisense oligonucleotides varies from cell to cell and one cannot use a delivery protocol that has been optimized for a different cell line without further optimization. Some cell lines such as Y1 allow for delivery of antisense oligonucleotides in the absence of a lipid carrier [27]; however, higher doses of oligonucleotides have to be applied (1–10 μM). There are a number of commercially available lipid carriers such as lipofectin (Gibco-BRL, Gaithersburg, MD), the pFx series from Invitrogen or the Tfx series (Promega, Madison, WI). We have not yet found a simple way to predict which lipid carrier performs the best for a given cell line. Before embarking on an antisense experiment with a specific cell line, we select the delivery vehicle using a fluorescence-tagged oligonucleotide. Fluorescence-tagged oligonucleotides are commercially available from most oligonucleotide synthesizing facilities.

Cells are plated at a density of 8×10^4 per well in a six-well plate 18 h before treatment. The labeled oligonucleotides (final concentration of 100 nM in the well) are mixed with the different lipids to be screened (in duplicates) at the DNA-to-lipid ratio recommended by the manufacturer and are applied onto six-well well dishes in a serum-free medium such as Optimem (Gibco-BRL) for 4 h. At this stage, the medium is replaced with fresh serum containing medium for 24 h. The medium is then removed, and the rate of incorporation of the fluorescence-tagged oligonucleotide is visually inspected using an inverted fluorescence microscope. Under optimal conditions 100% incorporation is attainable. Once the optimal lipid carrier is defined, a similar approach could be used to optimize the lipid–DNA ratio. We found that this assay is as good a predictor of the delivery of the oligonucleotide in antisense experiments as using DNMT1 mRNA levels as an endpoint.

4. Determining the potency and efficacy of antisense DNMT1 oligonucleotides

Once the optimal lipid carrier has been identified, it is critical to establish the EC_{50} and efficacy of the antisense inhibitor in the cell line of interest. As the predicted mechanism of action of antisense oligonucleotides is reduction of DNMT1 mRNA levels, it is critical to confirm dose-dependent inhibition of DNMT1 mRNA levels as a primary endpoint.

Cells are plated on 100-mm tissue culture dishes at a density of 10×10^5 cells per plate in triplicate per each concentration point. Eighteen hours after plating, the

medium is aspirated, the cells are rinsed with 5 ml of phosphate-buffered saline (PBS) and are then incubated with 6 ml OPTIMEM medium (Gibco-BRL) containing a mixture of either the antisense or control oligonucleotides (ranging from 1 to 100 nM) with the lipid carrier, which is prepared as follows. For A549 cells we add 37.5 μl of lipofectin (Gibco-BRL), 1–100 nM of the indicated oligonucleotides, and 6 ml of Optimem (Gibco-BRL) and leave the mixture at room temperature for 15 min. The transfection mixture is replaced with regular growth medium after 4 h. The treatment is repeated an additional time after 24 h. After 72 h the cells are harvested and counted.

4.1. Quantification of DNMT1 mRNA by RNase protection assay

RNA is prepared by any of the published standard procedures. We routinely prepare both nuclear and cytosolic fractions from the same cellular pellet as described below. RNA is extracted from the cytosolic fraction with the RNAzol preparation method using either commercially available kits (BRL), the method described by Chomczynski and Sacchi [37], or the guanidine thiocyanate method described by Chirgwin et al. [38]. DNMT1 mRNA could be quantified by Northern analysis; however, we have found that since effective oligonucleotide delivery requires that a low density of cells is used, the amount of RNA prepared from each point is too low to allow for detection of DNMT1 mRNA by Northern blot analysis. We have previously described an RNase protection assay [39] for detecting and quantifying DNMT1 [39] mRNA; competitive PCR assays for DNMT1 mRNA were also described [40].

Different fragments of DNMT1 cDNA or gene could be used as templates for riboprobe synthesis. For mouse cell lines, we use a 700-bp *Hin*dIII–*Bam*HI genomic fragment encoding the third and fourth exons of mouse DNMT1 subcloned in a SK plasmid downstream of a T7 promoter in the antisense orientation (Stratagene, La Jolla, CA) as described in [39]. For human cell lines we use a 300-bp *Xba*I genomic fragment bearing the third and fourth exons of human DNA MeTase mRNA similarly subcloned in SK [41]. A ^{32}P-labeled riboprobe is synthesized using the Ambion Maxiscript probe synthesis kit according to the manufacturer's protocol. The reaction mixture (20 μl) contains 0.3 μg of the linearized plasmid (bearing the 700-bp *Hin*dIII–*Bam*HI fragment or the 300-bp *Xba*I fragment), 5 μl[α-^{32}P]UTP (Amersham, 800 Ci/mmol) 0.5 mM of each of the other three ribonucleotides, 2 μl 10× transcription buffer (400 mM Tris–HCl, pH 7.6, 60 mM $MgCl_2$, 20 mM spermidine, 100 mM NaCl), 1 μl of RNase inhibitor (10 U/ml) (Boehringer or Ambion), 10 mM dithiothreitol mM (DTT), and 1 μl of T7 polymerase (10 U/μl). The synthesis is performed at 25 °C for 1 h. The quality of the synthesis is assessed by scintillation counting of the TCA-precipitated counts. A reasonable synthesis yields 1–2 × 10^6 cpm/μl. To normalize the signal to the amount of total RNA per sample, we synthesize an 18S ribosomal RNA riboprobe using a linear plasmid bearing 18S ribosomal RNA complementary sequences, provided in the Ambion RNase protection kit. Synthesis of 18S riboprobe is performed similarly to the synthesis of the DNMT1 probe except that cold UTP is added to the final concentration of 0.15 mM. Following synthesis of the labeled RNA, the plasmid DNA is digested by incubating the reaction mixture with 1 μl of RNase-free DNase (Boehringer, 10 U/μl) at 37 °C for 30 min. This step is critical, as remaining plasmid DNA hybridizes with the riboprobe and confounds the interpretation of the results. We have found that most technical difficulties in RNase protection assays could be traced to poor DNase digestion. DNase preparations tend to lose their activity even at −20 °C and it is advisable to replace the preparation when ambiguous hybridizations areseen in the RNase protection assay.

Following DNase digestion, the riboprobes are ethanol precipitated, washed with 70% ethanol and resuspended to final concentrations of 1 × 10^5 cpm/μl for the DNMT1 probe and 2 × 10^4/μl for the 18S riboprobe.

The different RNA samples (3 μg) are mixed with either 1 μl of the DNMT1 probe by itself (to determine the exact positions of the DNMT1 protected fragments) or with both 1 μl of 18S RNA and 1 μl of DNMT1 probes (to allow for normalizing the DNMT1 signal to the amount of total RNA per lane) in a hybridization buffer. The hybridization solution contains, in addition to the probes, 4 μl of 10× Pipes buffer (400 mM Pipes, Sigma, at pH 6.7; 4 M NaCl, 10 mM EDTA), 20 μl of formamide, and doubly distilled H_2O in a total reaction volume of 40 μl. The samples are incubated at 95 °C for 5 min, which is followed by an 18-h incubation at 50 °C. We use the following controls for each assay: 10 μg of tRNA (from yeast, Boehringer) hybridized with either of the probes to control for nonspecific hybridization and intact probes (that are not subjected to RNase digestion) to verify complete synthesis of the riboprobe. Following hybridization, the samples are subjected to RNase digestion with RNase A and T1 in a 300-μl RNase buffer containing 300 mM NaCl, 10 mM Tris–HCl, pH 7.6, 5 mM EDTA, 2 μl of RNase A (10 μg/μl, Boehringer), and 1.2 μl of RNase T1 (1.7 μg/μl) for 20 min at room temperature. The RNase solution is prepared fresh. (The buffer is kept at room temperature and the concentrated RNase solutions are kept at −20 °C.) The reaction is then terminated by adding 20 μl of a proteinase K (10 μg/μl), 10% sodium dodecyl sulfate (SDS) solution. The reaction mixture is vortexed and incubated for 15 min at 37 °C and the RNA is purified from contaminating proteins by phenol extraction, which is followed by standard ethanol

precipitation. We add 6 μg of tRNA to each sample to facilitate precipitation. The pellet is resuspended in a solution of 1 μl doubly distilled H_2O and 9 μl of formamide containing sequencing loading dye, boiled for 3 min, chilled on ice, and loaded on a 6% polyacrylamide urea sequencing gel. We use ^{32}P-labeled Century RNA markers (Ambion) as size markers (synthesis of the marker riboprobes is performed as recommended by the manufacturer). The gels are exposed either to autoradiography or a phosphorimaging plate and the signal obtained for DNMT1 is normalized to the signal obtained for the 18S ribosomal RNA probe.

4.2. Determining DNMT activity and protein levels in nuclear extracts prepared from antisense-treated cells

The preferred substrate for DNMT1 is hemimethylated DNA [42]. We therefore use a synthetic hemimethylated oligonucleotide duplex as a substrate for DNMT1. Methylated oligonucleotides could be synthesized by most commercial oligonucleotide facilities since the methylcytosine phosphoramidate is commercially available. We use the following sequence: 5′ GAT C_mGCC_m $GATGC_mGC_mGAATC_mGC_mGATC_m$ $GATGC_m$ GAT 3′; the complementary strand is not methylated [43]. To prepare nuclear extracts, we resuspend pelleted cells in buffer A (100 μl/1 × 10^6 cells) which contains 10 mM Tris–HCl, pH 8.0, 1.5 mM $MgCl_2$, 5 mM of KCl, 0.5 mM DTT, 0.5 mM (phenylmethylsulfonyl fluoride PMSF, Boehringer), and 0.5% of the detergent Nonidet P-40 (BDH). The pellets are homogenized in the buffer using a 1-ml Eppendorf tip, mixed gently by stirring, and incubated on ice for 15 min. The suspension is centrifuged in a microfuge at 1200 rpm for 15 min. The supernatant fraction is removed for RNA extraction using the RNAzol protocol (BRL) [37]. The nuclear pellets are washed once in buffer A and resuspended in 30 μl of a salt extraction buffer containing 20 mM Tris–HCl, pH 8.0, 25% glycerol, 1.5 mM $MgCl_2$, 0.5 mM PMSF, 0.2 mM EDTA, 0.5 mM DTT, and 0.4 M NaCl. The mixture is stirred gently and left on ice for 15 min. The nuclear extract is separated from the nuclear pellet by maximal speed centrifugation in a microfuge for 30 min at 4 °C. The nuclear extract is removed and the nuclear pellet is resuspended in 0.5 ml of DNA extraction buffer (10 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 10 mM EDTA, 100 U/ml proteinase K (BRL), and 10% SDS) for DNA extraction following standard protocols [44]. Nuclear extracts could be kept at −80 °C for a number of weeks without significant loss of DNMT1 activity.

DNMT1 assays are performed under initial rate conditions (0–3 h) using 3 μg of nuclear extract. Each determination is performed in triplicate in either the presence or absence (as a control for non-DNA-dependent incorporation of label) of 0.2 μg of the hemimethylated substrate in a total volume of 30 μl of the following reaction mixture: 15 μl of 2× Met buffer (40 mM Tris–HCl, pH 7.6, 50% glycerol, 20 mM EDTA, 0.4 mM PMSF, 40 mM (2-mercaptoethanol), 1 μCi of *S*-[methyl-^{3}H]adenosyl-L-methionine (Amersham, 78.9 Ci/mmol), and doubly distilled H_2O. Following incubation, the reaction mixtures are transferred into a tube containing 10% TCA and 20 μg of carrier DNA. After 15 min incubation on ice, the TCA mixture is filtered through GF/c filters (Fisher), the filters are washed twice with cold TCA, and the rate of incorporation of methyl groups into hemimethylated DNA is determined by scintillation counting and subtracting the counts obtained in the absence of hemimethylated DNA per sample. The activity is expressed as dpm methyl incorporated per microgram or milligram of nuclear extracts. We have observed up to >90% inhibition of DNMT1 activity in cells treated with 100 nM DNMT1 antisense oligonucleotides for 3 days.

To determine that the DNMT1 protein is reduced following antisense treatment, 50 μg of nuclear extract is resolved on 5% SDS–polyacrylamide gel electrophoresis (PAGE), transferred onto poly (vinylidene difluoride) membrane (Amersham), and subjected to immunodetection for DNMT1 according to standard protocols using a polyclonal antibody raised against a peptide sequence consisting of amino acids 1107–1125 of the mouse DNMT1 or 1101–1119 of the human DNMT1 protein [27]. The signal is visualized using a standard enhanced chemiluminescence kit (Amersham). To normalize the signal obtained for DNMT1 to the total protein transferred per lane, the membrane is subjected to amido black staining according to standard protocols [45] and the intensity of signals is determined by scanning densitometry.

4.3. Determining the state of global genomic DNA methylation

Once a specific dose–response relationship has been established between the antisense inhibitor and DNMT1 mRNA and protein levels, it is important to determine whether the treatment results in a global inhibition of DNA methylation. Hypomethylation will only occur only if replication proceeds in the presence of low levels of DNMT1 activity. However, if inhibition of DNMT1 results in an early inhibition of DNA replication as we have observed [18], the inhibition in DNA methylation might be initially very limited. Since the known mammalian DNMT1 specifically methylates CG dinucleotide sequences, it is critical to use a method that focuses on these sequences. We are using a modification [46] of a previously published nearest-neighbor analysis [47] to assay methylation specifically at CG dinucleotide sequences. The method is based on introducing a single labeled nucleotide, such as [α-^{32}P]dGTP, into DNA by nick translation, using DNase I to generate nicks and

DNA polymerase I to catalyze the replacement of the dGMP 3′ to the nick with a labeled [^{32}P]dGMP. The labeled phosphate group at the 5′ of the labeled nucleotide forms a phosphodiester bond with its 5′ neighbor, thus positioning a labeled phosphate at its 3′. Using a nuclease that cleaves the DNA to mononucleotides 3′ to the phosphate group will result in labeling of all of the 5′ neighbors of the labeled [^{32}P]dGMP. The different 5′ neighbors of G including C and ^{methyl}C are separated on thin-layer chromatography (TLC) and visualized by autoradiography (Fig. 1). The relative intensity of the dCMP and methyl dCMP are determined by either scraping the relevant spots and liquid scintillation counting, by scanning, or by phosphorimaging.

The protocol used currently in our laboratory is as follows: 100 ng of DNA is extensively vortexed to reduce viscosity which inhibits DNase activity. The DNA is added to a 10-μl reaction volume containing 1.5 μl of 10× nick translation buffer (0.5 M Tris–HCl, pH 7.4, 50 mM $CaCl_2$, 14 mM 2-mercaptoethanol)and 1.5 μl of DNase I (RNase-free DNase from Boehringer, 10 U/μl freshly diluted 1:100 in water). The reaction is incubated for 15 min at 37 °C and then chilled on ice. It is critical to maintain equivalent DNase nicking conditions for all samples. The method is dependent on randomly distributed nicks. Excess DNase activity results in double-stranded cleavage of DNA, whereas poor preparations of DNase result in limiting activity of DNase and selectivity of nicks. Following nicking of the DNA with DNase I, DNA polymerase I (Boehringer, 5 U/μl) and 1 μl of [α-^{32}P]dGTP (Amersham, 3000 Ci/mmol) are added to the reaction mixture which is then incubated for 15 min at 30 °C. The reaction is terminated by adding 30 μl of a Tris–EDTA solution (10 mM Tris–HCl, H 7.6, EDTA 10 mM). The labeled DNA is separated from nonincorporated radioactivity by passing it through a Sephadex G-50 spin column (Pharmacia). Eight microliters of the labeled DNA is digested to 3′ mononucleotides in a reaction mixture containing 1 μl of 10× micrococcal nuclease buffer (0.25 M Tris–HCl, pH 8.0, 10 mM $CaCl_2$) and 1 μl of micrococcal nuclease (Pharmacia, resuspended in doubly distilled H_2O to 150 U/μl) for 8 h at 37 °C. Three microliters of the reaction mixture is spotted onto cellulose TLC plates (Merck 13255 Cellulose without fluorescent indicator) and developed in an isobutyric acid:NH_3:H_2O (66:1:20) solvent. One microliter of cold unlabeled standards of each of the five monodeoxynucleotides (5 mg/ml) (Sigma) is loaded on the chromatograph to mark the relative migration of the different mononucleotides (the cold standards could be visualized by shining short-wave UV light on the plate). The chromatograms are exposed to either radioactivity or a phosphorimaging plate.

One critical point is to identify specific sequences that are differentially demethylated. Once candidates or potential candidates are identified, such as tumor suppressor genes, their state of methylation could be determined by either *MspI*/*Hpa*II Southern blot analysis [48], MSPCR [49], or bisulfite mapping [50]. These techniques are discussed elsewhere.

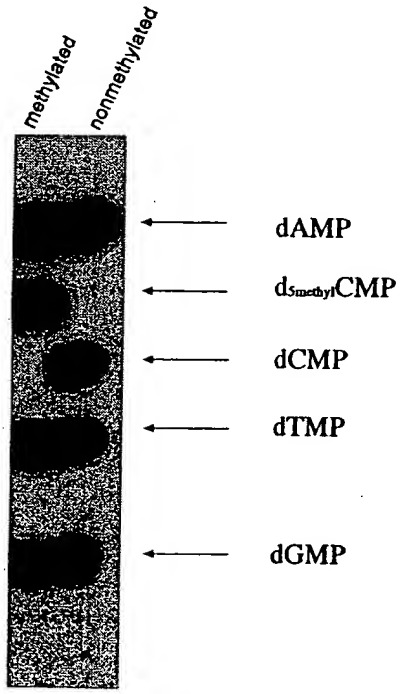

Fig. 1. Nearest-neighbor analysis of the state of methylation of CpG dinucleotides. A plasmid SK-methylated with the CpG DNA methyltransferase mSssI and an unmethylated SK plasmid are labeled with [α-^{32}P]dGTP, digested to 3′-mononucleotides and separated by TLC. An autoradiogram of the TLC is shown. The position of each of the 3′-mononucleotides is indicated.

5. Determining the effects that DNMT1 inhibitors might have on tumorigenesis in culture and in animals

One of the best assays for tumorigenesis in culture is to determine anchorage-independent growth on soft agar. It has been previously shown that this assay is an excellent predictor of tumorigenesis in vivo [51] and we have observed similar results.

5.1. Soft agar assay

We treat our cell lines with DNMT1 antisense and control oligonucleotides in triplicate at a dose range of 10–100 nM as described above. Using this dose range, we routinely observe a dose-dependent inhibition of DNMT1 with the antisense oligonucleotide. After 72 h of oligonucleotide treatment, the cells are harvested and counted, and the number of viable cells is determined by trypan blue exclusion. One thousand cells are seeded in triplicate onto a six-well dish (Falcon) with 4 ml medium containing 0.33% agar solution at 37 °C as

follows: The cells (1×10^5) are transferred onto a tube containing 3 ml enriched medium (30% enrichment in serum concentration, we use 13% serum for cells requiring 10% serum). A 1.5% agar (BDH) solution is autoclaved and then transferred to 50 °C and kept at this temperature until it is added to the medium containing tubes (1 ml per tube). Keeping the agar at 50 °C is critical to prevent congealing before it is homogeneously mixed with the medium containing the cells. However, some cell lines are very sensitive to temperature and lower temperatures should be tried when inadequate results are obtained with the conditions proposed here. The 4 ml soft agar medium is layered onto a single well in a six-well plate (Falcon). The agar is left to solidify and then 5 ml of fresh regular medium is applied on top of the agar. Cells are fed with 2 ml of medium every 3 days. Colonies could be visualized in most cases after 2 weeks. The number of colonies per treatment is determined by visual examination using a light microscope at low power (×10). Colonies containing more than 10 cells are counted and the effect of the DNMT1 antisense on tumorigenesis of the cell of interest in vitro is evaluated.

To assess the effects that antisense inhibitors of DNMT1 might have on tumor growth, differentiation, and gene expression in vivo, as well as to assess the effects that inhibition of DNMT1 might have on normal tissues in vivo, murine models are used. Antisense oligonucleotides could be delivered in vivo and are bioavailable following either intraperitoneal (ip), intravenous (iv), or subcutaneous (sc) injections. The pharmacokinetics of one DNMT1 antisense oligonucleotide has been recently analyzed in mouse models [52]. Three classes of models are available for studies using antisense DNMT1 oligonucleotides: first, mice strains bearing a genetic propensity to develop tumors such as mice bearing oncogenic transgenes or mutations in tumor suppressor genes [20]; second, mice bearing syngeneic implanted tumors such as the Y1 tumor in syngeneic LAF1 mice [27]; third, immunocompromized mice bearing human tumor cell lines xenografts. We and others have been routinely using the last two models. Each model has advantages and disadvantages. The first model is the closest to naturally occurring tumors and avoids artifacts resulting from continuous growth and selection pressures in culture. However, since tumors develop at different time points following birth and since tumors might not be initially externally visible, the starting point of treatment is relatively unpredictable and might vary from mouse to mouse. Treatment might have to be applied for a longer term to address these uncertainties. This increases the number of animals that need to be used to obtain statistical significance, which is especially relevant in the case of antisense oligonucleotides which are costly. The advantage of the syngeneic model is that animals with an intact immune system are used. This is important since the immune response is an important component in the response to tumors that have undergone hypomethylation treatment. In addition, the systemic effects ofantisense DNMT1 treatment could be evaluated since a mouse-specific DNMT1 antisense oligonucleotide is used in these experiments. The disadvantage of the model is that it does not use a human cancer model. We are routinely using both models in our laboratory.

There are two main ways to implant tumor cell lines in mice. The first method involves injecting cultured cancer cell lines in the flank or in any other organs. The other method is to serially passage a tumor cell line in mice, slice the tumors, and implant tumor fragments of a defined size in the flank. The advantage of the latter method is that tumors are implanted at a defined size, it avoids artifacts of tissue culture conditions, and the tumors maintain some of the tissue organization. We are using both protocols in our laboratory.

To study the effects of DNMT1 inhibition in a syngeneic mouse tumor model, we inject into the flank of 10 LAF1 mice (Charles River) per treatment group 1×10^6 freshly harvested Y1 cells resuspended in a 100-μl phosphate-buffered saline (PBS) solution. A typical experiment includes an antisense group, a scrambled or mismatch control, and a PBS vehicle control. We initiate treatment 3 days after implantation with either intraperitoneal, intravenous, or subcutaneous injections daily with 2 mg/kg oligonucleotide dissolved in 100 μl of PBS. Our standard experiment extends for 30 days. We follow the following endpoints daily: tumor diameter (the volume of the tumors is calculated as in Ref. [53]) and animal weights to assess general toxicity. At the termination of the experiment, the animals are sacrificed, and the tumors are removed and weighed. Comparison of the average weights of the tumors indicates the effect that the oligonucleotides have on tumor growth. Tumor tissue as well as other animal tissue could be processed for histology and protein analyses; RNA and DNA could be extracted to determine effects on DNMT1 expression and DNA methylation as described above.

An alternative method is used in our laboratory for human xenografts. One million human non-small lung carcinoma cells A549 (ATCC: Marassus, VA CCL 185) are injected into the flank of athymic BALB/c nude mice (Charles River) (resuspended in 100 μl of PBS). Once the tumors are established, they are harvested and cut into 25-mg fragments which are injected into the flank of other athymic nude mice using a 13-gauge trocar needle. The mice are monitored for the presence and size of tumors. Once the tumors have reached a volume of 0.1 cm^3 they are injected sc daily with either 2 mg/kg antisense, control oligonucleotides, or vehicle (100 μl PBS). Tumor size as well as animal weight is monitored throughout the experiment as discussed above.

6. Conclusions

DNMT1 is emerging as an important component of oncogenic pathways. Unraveling its role in different cancer models requires the use of specific inhibitors. The inhibitor that is most commonly used currently, 5-aza-CdR, exhibits cytotoxic side effects that confound the interpretation of the experimental data. DNMT1 antisense oligonucleotides have been developed that specifically reduce DNMT1 mRNA. These oligonucleotides can be used to determine whether DNMT1 plays a causal role in different cancer models, both in cell culture and in vivo. The specificity of these compounds could be determined by measuring the endpoints discussed in this paper. DNMT1 antisense oligonucleotides are also useful tools in determining the down- stream effectors of DNMT1 and identifying genes involved in cancer that are possibly controlled by methylation.

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